EMBO reports

Stimulation of glycolysis promotes cardiomyocyte proliferation after injury in adult zebrafish.

Ryuichi Fukuda, Rubén Marín-Juez, Hadil El-Sammak, Arica Beisaw, Radhan Ramadass, Carsten Kuenne, Stefan Guenther, Anne Konzer, Aditya Bhagwat, Johannes Graumann, and Didier Stainier
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Corresponding author(s): Didier Stainier (Didier.Stainier@mpi-bn.mpg.de) , Didier Stainier (Didier.Stainier@mpi-bn.mpg.de), Ryuichi Fukuda (ryuichi.fukuda@mpi-bn.mpg.de)

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 19th Dec 2019

Dear Didier,

Thank you for the submission of your research manuscript to our journal, which was now seen by two referees, whose reports are copied below.

As you can see, the referees express interest in the analysis. However, they also raise a number of concerns that need to be addressed to consider publication here. I find the reports informed and constructive, and believe that addressing the concerns raised will significantly strengthen the manuscript.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1. A data availability section providing access to data deposited in public databases is missing (where applicable).
2. Your manuscript contains statistics and error bars based on n=2 or on technical replicates. Please use scatter plots in these cases.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page with page numbers, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper. For more details on our Transparent Editorial Process, please visit our website:
https://www.embopress.org/page/journal/14693178/authorguide#transparentprocess
You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

4) a complete author checklist, which you can download from our author guidelines (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines ().

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: .

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) We would also encourage you to include the source data for figure panels that show essential data.

Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

8) Regarding data quantification, please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
Please note that error bars and statistical comparisons may only be applied to data obtained from

at least three independent biological replicates.
Please also include scale bars in all microscopy images.

9) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

10) Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see).

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Method) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Kind regards,

Deniz

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

The authors show by microscopy of marker gene expression that impaired glycolysis interferes with cardiomyocyte proliferation and therefore heart regeneration in zebrafish models of cardiac injury and rat cardiomyocytes.

Fig1b,c
Could the authors provide a control for the efficiency of 2-DG and DCA treatment in order to be able to judge the effect size of the proliferation and embryonic myosin expression defect?

Fig1 and Fig2 general:
Since a decrease in glycolysis and Pdk function is hitting very basic cellular pathways I'm not sure about the specificity of this effect: is it not expected that an intervention to a basic pathway would impair proliferation required for injury? Could a loss-of-function of for instance cell cycle control not have a similar effect? It would be helpful if the authors can comment on this especially in light of the Honkoop et al., 2018 study with which this study largely overlaps.

Also, it would be interesting to learn whether or not the authors think the proposed role of glycolysis is limited to the heart or if it can be applied to other tissues.

Fig3
Controls for a mock overexpression and overexpression levels are missing.

Fig4
Since the overexpression of PDK3 in rat cardiomyocytes seems to promote formation of membrane protrusions and proliferation and this is interpreted to be a potentially therapeutically relevant phenomenon, the manuscript would benefit from a more detailed phenotypization of cells after overexpression (e.g. by RNA-Seq): What are the downstream effects of the overexpression? How similar are the cells to wild type cells? Is there any negative impact on cell behavior expected or observed?

Referee #2:

The manuscript by Fukuda et al. reports on a link between cardiac metabolism and regeneration using zebrafish heart and neonatal rat cardiac myocytes as model systems.
The author first report on the observation that metabolisms shifts from oxydative phosphorylation to glycolysis in response to wounding in the border zone area. Pharmacological and genetic modulation of cardiac metabolism towards oxidative phosphorylation caused a decrease in proliferation, while the reverse was true when glycolysis was promoted. Glycolysis has been implicated for a long time in cancer and recently also in many forms of tissue regeneration including cardiac regeneration see for example a review by Magadum and Engel PPARβ/δ: Linking Metabolism to Regeneration. Int J Mol Sci. 2018 Jul 10;19(7). pii: E2013. Wang X, et al. TLR3 Mediates Repair and Regeneration of Damaged Neonatal Heart through Glycolysis Dependent YAP1 Regulated miR-152 Expression. Cell Death Differ. 2018;25(5):966-982. In this regard te manuscript lacks a bit of novelty. Although the specific experiments shown here have not been performed yet, the overall conclusions have also been drawn previously. While the data are convincing

1. Is the cell number really different? In some of the panels the different labelling indices are optically really not very convincingly different. It would therefore be advisable to have an independent way of quantifying the changes. In cases where genetic manipulations are employed, the labeled cells could be sorted and quantified.

2. I think the best experiment to test whether the experimental results in zebrafish can be extrapolated to mammals would be the use of infarcted neonatal hearts. The authors have chosen to utilize a neonatal rat cardiomyocyte model. While the Ki67 labelling is quit convincing, I would like to know whether the cell number has really changed in response to the treatments.

3. I am a bit skeptical about the wounding assay and the significance of the cell protusions, which are formed at higher frequency in the case of PDK3 expression. It would be good in addition to the stills to have also some time lapse data to specifically demonstrate the increased protusive activity in case of neonatal cardiac myocytes.

4. How is glycolysis mechanistically linked to cell cycle activity? What is known about it and does this apply to zebrafish and mammalian heart regeneration?

Minor:
1. Many of the panels contain arrowheads, which point to in my opinion single and double labeled cardiac myocytes. Please also include in the legend a statement what those arrowheads mean and review whether different symbols might be needed to distinguish differentially labeled cells.
2. I don't like the fact that the first figure mentioned in the paper is a supplemental figure.

Referee #3:

This study from Fukada and colleagues describes the role of a metabolic switch in cardiomyocytes during zebrafish heart regeneration. Cardiomyocytes use glucose during development as their primary source of energy but later switch to fatty acids during the maturation of the heart. During zebrafish heart regeneration, cardiomyocytes acquire a less differentiated phenotype and re-express embryonic markers. The authors wanted to determine whether this process of de-differentiation involves a reversion from OXPHOS to glycolysis. To this end, they analyzed the expression of glycolytic genes in hearts after cryoinjury. Interestingly, they found an upregulation of glycolytic genes in the border zone. Blocking glycolysis using chemical inhibitors reduced cardiomyocyte de-differentiation and proliferation. Next, the authors use mutant strains that promote the use of pyruvate via OXPHOS or anaerobic glycolysis. They found that proliferation in cardiomyocytes increased when anaerobic fermentation instead of OXPHOS consumed pyruvate. The authors used tissue-specific manipulations and confirmed their previous findings. Lastly, the authors demonstrated that this mechanism is conserved by manipulating pyruvate metabolism in rat neonatal cardiomyocytes and measuring proliferation in different conditions.

Overall, I find this work of conceptual interest and believe that it provides new relevant information to the regeneration community. A preprint from the Bakkers lab from a few months ago tackled the same question, but in this manuscript, the authors not only provide correlative evidence, but they also test experimentally their hypothesis using genetic tools. While I find the data convincing, the technical quality of the data is somehow lower than the standard in the zebrafish heart regeneration community. Additionally, I believe that the manuscript would benefit from revising the text in depth.

Major concerns
(1) This article would benefit enormously from re-writing several sections to correct some oversimplifications, conceptual mistakes, and make the text much more accessible for the non-expert reader. On several occasions, the authors should include explicit statements about their results or the consequences of the mutations that they report. Please consider the following examples:
• In the introduction, the authors say that "Glucose is converted into pyruvate by pyruvate kinase M1/2". This phrase is a considerable oversimplification that may confuse readers. I don't believe the authors should list the ten reactions that transform glucose in pyruvate, but they should rephrase their text so that it is not misleading.
• The next sentence in the introduction claims that "pyruvate is further utilized via glycolysis or oxidative phosphorylation to produce ATP". The transformation of glucose to pyruvate is already called glycolysis. Pyruvate can be then processed in the mitochondria during oxidative phosphorylation or, alternatively, processed to lactate in anaerobic fermentation. Again, I feel that these details should be corrected in the text.
• "Pdk inhibits PDC function, resulting in promotion of pyruvate metabolism in glycolysis". This sentence is also confusing. By inhibiting the transformation of pyruvate in Acetyl Coenzyme A, PDK induces the transformation of pyruvate in lactate, via anaerobic fermentation.
• When introducing the mutants that the authors use in their regeneration studies, they should be very explicit about what these mutations are doing. For example, in pages 4 and 5, the authors should state what is the expected use of pyruvate in these mutants (OXPHOS or anaerobic fermentation). They should also provide an interpretation of the phenotype of the pkma mutants at the end of the first paragraph on page 5.

(2) On two occasions, the authors discuss their results from a submitted manuscript. I feel that they should either remove any mention to these results or post them in a pre-print server so that we can have access to them.
(3) Perhaps because of the "Report" nature of this manuscript, the authors have not discussed their findings or put them in the general context of heart regeneration. How do they interpret the biological meaning of their results? They should speculate whether the morphological defects reported in mitochondria in border zone cardiomyocytes could contribute to this phenotype.
(4) The authors should also discuss the fact that in the injured mammalian heart, a switch from OXPHOS to glycolysis is also common, despite the lack of regeneration.

I have two additional comments that I feel would increase the strength of this manuscript substantially. While I do not believe these experiments should condition the acceptance of this manuscript, I think they could be completed in a relatively short time (~60 days), and the authors have all the reagents and lines at hand.
• The authors have focused in the very early phases of regeneration, which is fine to analyze cardiomyocyte proliferation. However, we do not really know whether these mutations or transgenic manipulations affect regeneration in the long term. Do the pkma2 mutants regenerate the myocardial wall at later stages (~60 days post-injury)?
• Is the switch to anaerobic glycolysis sufficient to induce cardiomyocyte proliferation in the absence of any injury?

Minor comments
(1) Why do the authors use pkm2a-/- pkmb+/- instead of just pkm2a -/- animals? Is the phenotype only apparent in this genetic situation?
(2) Please specify how many sections per heart have been analyzed to calculate the cardiomyocyte

proliferation index. It is standard in the field to quantify three individual sections and average them to obtain the value per animal.
(3) Please clarify the treatment of control animals in Figure 3. Are control animals non-transgenic siblings that are also heat-shocked?
(4) Arrows in Figure 1B, panel DCA, are not pointing to any nuclei.

Referee #1:

The authors show by microscopy of marker gene expression that impaired glycolysis interferes with cardiomyocyte proliferation and therefore heart regeneration in zebrafish models of cardiac injury and rat cardiomyocytes.

We thank the reviewer for her/his constructive comments.

Fig1b,c
Could the authors provide a control for the efficiency of 2-DG and DCA treatment in order to be able to judge the effect size of the proliferation and embryonic myosin expression defect?

We have now examined lactate levels to assess the effects of 2-DG and DCA treatments on glycolysis (Fig. EV1a). These data indicate that those treatments significantly decrease lactate levels.

Fig1 and Fig2 general:
Since a decrease in glycolysis and Pdk function is hitting very basic cellular pathways I'm not sure about the specificity of this effect: is it not expected that an intervention to a basic pathway would impair proliferation required for injury? Could a loss-of-function of for instance cell cycle control not have a similar effect? It would be helpful if the authors can comment on this especially in light of the Honkoop et al., 2018 study with which this study largely overlaps.

We agree with the reviewer that the decrease of a basic pathway is expected to impair proliferation required for regeneration. However, it has been shown that glycolysis plays an important role in the proliferation of cancer cells (Liberti and Locasale, 2016), endothelial cells (De Bock et al., 2013; Wilhelm et al., 2016) and neural progenitor cells (Zheng et al., 2016). Cardiomyocyte-specific genetic manipulations in mice indicate that overexpression of *Pkm2*, a key glycolytic gene, promotes glycolysis and cardiomyocyte proliferation, while loss of *Pkm2* decreases cardiomyocyte proliferation (Magadum et al., 2020). Consistently, PDK overexpression promotes ovarian cancer proliferation (Wang et al., 2019). During zebrafish cardiac regeneration, the data in Honkoop's paper show that glycolytic genes are upregulated in regenerating cardiomyocytes. Our data show that cardiomyocyte-specific activation of glycolysis increases the number of proliferating cardiomyocytes following injury. These lines of evidence suggest that glycolysis promotes cardiomyocyte proliferation in both mice and zebrafish.

Regarding the effects of loss-of-function of cell cycle regulators, loss of Mps1, a mitotic checkpoint kinase (Poss et al., 2002), and inhibition of Plk1, a regulator of cell cycle progression (Jopling et al., 2010), lead to a decrease in cardiomyocyte proliferation during zebrafish heart regeneration.

We have now performed RNA-seq analysis of PDK3 overexpressing rat cardiomyocytes, and the data indicate that PDK3 overexpression in cardiomyocytes leads to increased levels of genes encoding factors that promote cell cycle and DNA replication (Figs. 4d-f, Figs. EVf, g). These data suggest that enhanced glycolysis by PDK3 overexpression promotes the cell cycle at least in part by regulating gene expression. However, determining the specific mechanisms of how glycolysis promotes cardiomyocyte proliferation during cardiac regeneration will require extensive further studies.

Also, it would be interesting to learn whether or not the authors think the proposed role of glycolysis is limited to the heart or if it can be applied to other tissues.

Glycolysis also regulates endothelial cells (De Bock et al., 2013; Wilhelm et al., 2016), neural progenitor cell proliferation (Zheng et al., 2016) and skeletal muscle regeneration (Wagner et al., 1976).

Fig3
Controls for a mock overexpression and overexpression levels are missing.

In these experiments, the controls consist of *Tg(hsp70l:LOXP-STOP-LOXP-pdha1aSTA-T2A-mCherry)* and *Tg(hsp70l:LOXP-STOP-LOXP-pdk3b-T2A-mCherry)* animals without *Tg(myl7:Cre-ERT2)* and treated with tamoxifen and heat shock.

The expression levels of the transgenes in *Tg(hsp70l:LOXP-STOP-LOXP-pdha1aSTA-T2A-mCherry); Tg(myl7:Cre-ERT2)* and *Tg(hsp70l:LOXP-STOP-LOXP-pdk3b-T2A-mCherry); Tg(myl7:Cre-ERT2)* after tamoxifen and heat shock treatments were examined by mCherry signal (Figs. EV3a, b). Since we do not have antibodies against zebrafish Pdha1a and Pdk3b, we cannot assess their expression levels.

Fig4
Since the overexpression of PDK3 in rat cardiomyocytes seems to promote formation of membrane protrusions and proliferation and this is interpreted to be a potentially therapeutically relevant phenomenon, the manuscript would benefit from a more detailed phenotypization of cells after overexpression (e.g. by RNA-Seq): What are the downstream effects of the overexpression?

We have now performed RNA-seq analysis of PDK3 overexpressing rat cardiomyocytes, and the data indicate that PDK3 overexpression in cardiomyocytes leads to increased levels of genes encoding factors that promote cell cycle and DNA replication (Figs. 4d-f, Figs. EVf, g). These data suggest that enhanced glycolysis by PDK3 overexpression promotes the cell cycle at least in part by regulating gene expression.

How similar are the cells to wild type cells? Is there any negative impact on cell behavior expected or observed?

We have previously reported that PDK3 overexpression led to an increase in membrane protrusions in rat neonatal cardiomyocytes (Fukuda et al., eLife, 2019). These data are consistent with our observations in the scratched assay (Fig. 4c and Movie EV1, Movie EV3). We did not see a significant increase in cell death in PDK3 overexpression samples following DAPI and cardiac troponin I staining (Fig. 4b).

Referee #2:

The manuscript by Fukuda et al. reports on a link between cardiac metabolism and regeneration using zebrafish heart and neonatal rat cardiac myocytes as model systems.
The author first report on the observation that metabolisms shifts from oxydative phosphorylation to glycolysis in response to wounding in the border zone area. Pharmacological and genetic modulation of cardiac metabolism towards oxidative phosphorylation caused a decrease in proliferation, while the reverse was true when glycolysis was promoted. Glycolysis has been implicated for a long time in cancer and recently also in many forms of tissue regeneration including cardiac regeneration see for example a review by Magadum and Engel PPARβ/δ: Linking Metabolism to Regeneration. Int J Mol Sci. 2018 Jul 10;19(7). pii: E2013. Wang X, et al. TLR3 Mediates Repair and Regeneration of Damaged Neonatal Heart through Glycolysis Dependent YAP1 Regulated miR-152 Expression. Cell Death Differ. 2018;25(5):966-982. In this regard te manuscript lacks a bit of novelty. Although the specific experiments shown here have not been performed yet, the overall conclusions have also been drawn previously. While the data are convincing

We thank the reviewer for her/his constructive comments.

Glycolysis has been implicated for a long time in cancer and recently also in many forms of tissue regeneration including cardiac regeneration see for example a review by Magadum and Engel PPARβ/δ: Linking Metabolism to Regeneration. Int J Mol Sci. 2018 Jul 10;19(7). pii: E2013. Wang X, et al. TLR3 Mediates Repair and Regeneration of Damaged Neonatal Heart through Glycolysis Dependent YAP1 Regulated miR-152 Expression. Cell Death Differ. 2018;25(5):966-982.

We have now referred to these papers in the discussion part of the revised manuscript.

1. Is the cell number really different? In some of the panels the different labelling indices are optically really not very convincingly different. It would therefore be advisable to have an independent way of quantifying the changes. In cases where genetic manipulations are employed, the labeled cells could be sorted and quantified.

We have now also examined cardiomyocyte proliferation by carrying out phospho histone 3 immunostaining as another proliferation marker (Figs. EV1b, EV2d, EV2g, EV3c, EV3d), and the results are consistent with our data using PCNA immunostaining. Unfortunately, sorting cardiomyocytes from adult zebrafish hearts is not a very efficient or reliable procedure.

2. I think the best experiment to test whether the experimental results in zebrafish can be extrapolated to mammals would be the use of infarcted neonatal hearts. The authors have chosen to utilize a neonatal rat cardiomyocyte model. While the Ki67 labelling is quit convincing, I would like to know whether the cell number has really changed in response to the treatments.

We have done some cell counting and found that PDHA1STA overexpression led to a decrease in the number of cardiomyocytes (Fig. EV4d). However, PDK3 overexpression did not lead to a significant change in cardiomyocyte numbers (Fig. EV4e). We have also carried out phospho histone 3 immunostaining, and found that PDK3 overexpression led to an increase in the number of phospho histone 3+ cardiomyocytes (Fig. EV4c). These data suggest that PDK3 overexpression affects cell cycle progression but does not significantly change cardiomyocyte numbers.

3. I am a bit skeptical about the wounding assay and the significance of the cell protusions, which are formed at higher frequency in the case of PDK3 expression. It would be good in addition to the stills to have also some time lapse data to specifically demonstrate the increased protusive activity in case of neonatal cardiac myocytes.

We have now performed time lapse imaging (Movies EV1-3), and found that the results are consistent with our previous observations.

4. How is glycolysis mechanistically linked to cell cycle activity? What is known about it and does this apply to zebrafish and mammalian heart regeneration?

It has been shown that glycolysis plays an important role in the proliferation of cancer cells (Liberti and Locasale, 2016), endothelial cells (De Bock et al., 2013; Wilhelm et al., 2016) and neural progenitor cells (Zheng et al., 2016). Cardiomyocyte-specific genetic manipulations in mice indicate that overexpression of *Pkm2*, a key glycolytic gene, promotes glycolysis and cardiomyocyte proliferation, while loss of *Pkm2* decreases cardiomyocyte proliferation (Magadum et al., 2020). Consistently, PDK overexpression promotes ovarian cancer proliferation (Wang et al., 2019). During zebrafish cardiac regeneration, the data in Honkoop's paper show that glycolytic genes are upregulated in regenerating cardiomyocytes. Our data show that cardiomyocyte-specific activation of glycolysis increases the number of proliferating cardiomyocytes following injury. These lines of evidence suggest that glycolysis promotes cardiomyocyte proliferation in both mice and zebrafish.

We have now performed RNA-seq analysis in PDK3 overexpression rat cardiomyocytes, and found that genes encoding factors which promote cell cycle and DNA replication were upregulated in PDK3 overexpressing cardiomyocytes (Figs. 4d-f, EV4f, EV4g). These data suggest that enhanced glycolysis by PDK3 overexpression regulated genes regulating cell cycle progression. However, the specific mechanism of how glycolysis promotes cardiomyocyte proliferation during cardiac regeneration requires further studies.

Minor:
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We have now improved the legends.

2. I don't like the fact that the first figure mentioned in the paper is a supplemental figure.

We have now moved these data to figures 1a and b.

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Overall, I find this work of conceptual interest and believe that it provides new relevant information to the regeneration community. A preprint from the Bakkers lab from a few months ago tackled the same question, but in this manuscript, the authors not only provide correlative evidence, but they also test experimentally their hypothesis using genetic tools. While I find the data convincing, the technical quality of the data is somehow lower than the standard in the zebrafish heart regeneration community. Additionally, I believe that the manuscript would benefit from revising the text in depth.

We thank the reviewer for her/his positive and constructive comments.

Major concerns
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M1/2". This phrase is a considerable oversimplification that may confuse readers. I don't believe the authors should list the ten reactions that transform glucose in pyruvate, but they should rephrase their text so that it is not misleading.

We have now changed the sentence in the revised manuscript and now it reads:

During glycolysis, glucose is converted to pyruvate through several intermediates, and then pyruvate is further utilized to produce lactate, via anaerobic fermentation, or acetyl-coenzyme A (acetyl-CoA) via oxidative phosphorylation (OXPHOS) to produce ATP (Takubo et al., 2013).

• The next sentence in the introduction claims that "pyruvate is further utilized via glycolysis or oxidative phosphorylation to produce ATP". The transformation of glucose to pyruvate is already called glycolysis. Pyruvate can be then processed in the mitochondria during oxidative phosphorylation or, alternatively, processed to lactate in anaerobic fermentation. Again, I feel that these details should be corrected in the text.

We have now changed the sentence as above.

• "Pdk inhibits PDC function, resulting in promotion of pyruvate metabolism in glycolysis". This sentence is also confusing. By inhibiting the transformation of pyruvate in Acetyl Coenzyme A, PDK induces the transformation of pyruvate in lactate, via anaerobic fermentation.

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We have now changed the text in pages 5 and 6 of the revised manuscript

(2) On two occasions, the authors discuss their results from a submitted manuscript. I feel that they should either remove any mention to these results or post them in a pre-print server so that we can have access to them.

This paper has now been published:
Fukuda, R., Aharonov, A., Ong, Y. T., Stone, O. A., El-Brolosy, M., Maischein, H. M., Potente, M., Tzahor, E., & Stainier, D. Y. (2019). Metabolic modulation regulates cardiac wall morphogenesis in zebrafish. eLife, 8, e50161.

(3) Perhaps because of the "Report" nature of this manuscript, the authors have not discussed their findings or put them in the general context of heart regeneration. How do they interpret the

biological meaning of their results? They should speculate whether the morphological defects reported in mitochondria in border zone cardiomyocytes could contribute to this phenotype.

We have now discussed this point as below and added it on pages 9 and 10 of the revised manuscript.

Our data indicate that increase of glycolytic activity by Pdk3 OE promotes cardiomyocyte dedifferentiation following injury. It was reported that in regenerating cardiomyocytes, mitochondria exhibit an immature structure and that the levels of mitochondrial gene expression are reduced (Honkoop et al., 2019), indicating lower mitochondrial activity. In addition, mitochondrial function increases during cardiomyocyte maturation in the developing heart (Menendez-Montes et al., 2016). Thus, reduced mitochondrial activity, and therefore reduced OXPHOS, may be important for cardiomyocyte dedifferentiation and proliferation during regeneration.

(4) The authors should also discuss the fact that in the injured mammalian heart, a switch from OXPHOS to glycolysis is also common, despite the lack of regeneration.

We have now discussed this point as below and added it on page 10 of the revised manuscript.

It has been shown that the levels of glycolytic enzymes also increase following cardiac ischemia and in failing hearts (Das et al., 1987; Doenst et al., 2013). However, adult mice do not exhibit robust cardiac regeneration. Recent data show that the upregulation of PKM2 levels following cardiac injury is limited and thus possibly insufficient to induce cardiomyocyte proliferation in an ischemic adult mouse model (Magadum et al., 2020), while Pkm2 overexpression induced cardiomyocyte proliferation after injury (Magadum et al., 2020). Together, these data suggest that the levels of glycolytic gene expression in mice following cardiac injury might be a limiting factor in terms of promoting cardiomyocyte proliferation. It will be interesting to further investigate the differences in metabolic changes between zebrafish and mouse cardiac injury models.

I have two additional comments that I feel would increase the strength of this manuscript substantially. While I do not believe these experiments should condition the acceptance of this manuscript, I think they could be completed in a relatively short time (~60 days), and the authors have all the reagents and lines at hand. • The authors have focused in the very early phases of regeneration, which is fine to analyze cardiomyocyte proliferation. However, we do not really know whether these mutations or transgenic manipulations affect regeneration in the long term. Do the pkma2 mutants regenerate the myocardial wall at later stages (~60 days post-injury)?

We have now examined *pkma2* mutants at 60 dpci. We performed Acid Fuchsin Orange G (AFOG) staining to examine scar size. Notably, *pkma2-/-; pkmb+/-* animals exhibited significantly larger scar area compared to that in *pkma2+/-; pkmb+/-* animals (Fig. 2e). However, *ppargc1a* mutants did not exhibit a significant difference in scar area (Fig. 2f). We also examined the effects of cardiomyocyte-specific metabolic modulation on scar size using our transgenic animals. We found that at 60 dpci *pdha1aSTA* overexpression in cardiomyocytes led to larger scar areas compared to control (Fig. 3f), but *pdk3b* overexpression did not affect scar size (Fig. 3g).

• Is the switch to anaerobic glycolysis sufficient to induce cardiomyocyte proliferation in the absence of any injury?

We have now examined *Tg(hsp70l:LOXP-STOP-LOXP-pdk3b-T2A-mCherry); Tg(myl7:Cre-ERT2)* animals treated with tamoxifen and heat shock in the absence of cardiac cryoinjury, and found no obvious increase in the number proliferating cardiomyocytes (Fig. EV3e).

Minor comments

(1) Why do the authors use pkm2a-/- pkmb+/- instead of just pkm2a -/- animals? Is the phenotype only apparent in this genetic situation?

We have now examined *pkma2-/-* animals and found that they also exhibit a decrease in PCNA+ and N2.261+ cardiomyocytes following injury; we have added these new data to the revised manuscript (Figs. EV2e, f).

(2) Please specify how many sections per heart have been analyzed to calculate the cardiomyocyte proliferation index. It is standard in the field to quantify three individual sections and average them to obtain the value per animal.

We examined three individual sections per heart in all relevant experiments, and have now included this important information in the Methods section in the revised manuscript.

(3) Please clarify the treatment of control animals in Figure 3. Are control animals non-transgenic siblings that are also heat-shocked?

In this experiment, tamoxifen and heat shock treated *Tg(hsp70l:LOXP-STOP-LOXP-pdha1aSTA-T2A-mCherry)* and *Tg(hsp70l:LOXP-STOP-LOXP-pdk3b-T2A-mCherry)* animals without *Tg(myl7:Cre-ERT2)* were used as controls.

(4) Arrows in Figure 1B, panel DCA, are not pointing to any nuclei.

We have now fixed this issue in the revised manuscript.

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1st Revision - Editorial Decision 30th Apr 2020

Dear Didier,

Thank you for submitting the revised version of your manuscript. It has now been seen by all of the original referees.

As you can see, the referees find that the study is significantly improved during revision and recommend publication. Before I can accept the manuscript, I need you to address some minor points below:

• Please provide 3-5 keywords for your study. These will be visible in the html version of the paper and on PubMed and will help increase the discoverability of your work.
• We noted that the information regarding data deposition is currently present in the Materials&Methods section. Please make a separate 'Data Availability Section' and move this information there.
• We realized that Figure 2F is currently not called out in the text.
• The movies need to be ZIPped with their legends. The legends need to be removed from the Article file.
• We noted that Fig EV1, EV2 and EV3 magnification inserts are missing scale bars.
• We realized that there is not sufficient distance between some microscopy panels (especially figure 3c and e).
• Our character limit for titles is 100 (including spaces) for technical reasons. Therefore please shorten the current title.
• Papers published in EMBO Reports include a 'Synopsis' to further enhance discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the key findings of the paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb.
• In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.
• Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your minor revision.

Kind regards,
Deniz
--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

The authors have addressed all my concerns.

Referee #2:

The authors of the manuscript by Fukuda et al. have carefully addressed my comments to the original manuscript. I have no further comments.

Referee #3:

The authors have addressed all my comments, and I evaluate the manuscript positively.

Max-Planck-Institute
for Heart and Lung Research
W.G. Kerckhoff-Institute

Dr. Deniz Senyilmaz Tiebe
Editor, *EMBO Reports*

May 12, 2020

Dear Deniz,

Thank you again for your help with our manuscript now entitled "**Stimulation of glycolysis promotes cardiomyocyte proliferation after injury in adult zebrafish**".

We have now changed the manuscript and the figures according to your comments.

Please find below our point-by-point response to your comments.

Thank you very much and best regards,

Ryuichi Fukuda and Didier Stainier

・Please provide 3-5 keywords for your study. These will be visible in the html version of the paper and on PubMed and will help increase the discoverability of your work.

Cardiac regeneration, Zebrafish, Glycolysis, Metabolism, Cardiomyocyte proliferation

・We noted that the information regarding data deposition is currently present in the Materials&Methods section. Please make a separate 'Data Availability Section' and move this information there.

We have now moved this information to the Data Availability section in the revised manuscript.

・We realized that Figure 2F is currently not called out in the text.

Figure 2F has now been called out in the revised manuscript.

・The movies need to be ZIPped with their legends. The legends need to be removed from the Article file.

The legends for the movies have now been removed from the revised manuscript and have been ZIPped with the movies.

・We noted that Fig EV1, EV2 and EV3 magnification inserts are missing scale bars.

Scale bars have been added in the revised figures.

・We realized that there is not sufficient distance between some microscopy panels (especially figure 3c and e).

We have changed the layout of Figs. 2, 3, EV1, EV2 and EV3.

・Our character limit for titles is 100 (including spaces) for technical reasons. Therefore please shorten the current title.

The title has been changed to "**Stimulation of glycolysis promotes cardiomyocyte proliferation after injury in adult zebrafish**".

・Papers published in EMBO Reports include a 'Synopsis' to further enhance discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the key findings of the paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb.
・In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.

We have included a synopsis and an image.

・Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

We have changed these points in the revised manuscript.

2nd Revision - Editorial Decision 20th May 2020

Dear Didier,

Thank you for submitting your revised manuscript. I have now looked at everything and all looks fine. Therefore I am very pleased to accept your manuscript for publication in EMBO Reports.

Congratulations on a very nice study!

Kind regards,

Deniz
--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Didier Stainier
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2019-49752V3

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was determined based on published literature.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We described it in the manuscript.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	No randomization was used.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done.
5. For every figure, are statistical tests justified as appropriate?	We described it in the manuscript.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We did not used any methods to assess it.
Is there an estimate of variation within each group of data?	No.

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Is the variance similar between the groups that are being statistically compared?	We did not assess it.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We describe this information in the Methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	We describe this information in the Methods section.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	We describe those information in the Methods section.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	All animal experiments were done under standard conditions in accordance with institutional (MPG) and national ethical and animal welfare guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	We describe this information in the Methods section.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	We describe this information in the Methods section.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	We did not use agents or toxins from this list.